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15-Deoxy-Δ-12-14-PGJ_2 Regulates Apoptosis Induction and Nuclear Factor-κB Activation Via a Peroxisome Proliferator-Activated Receptor-γ–Independent Mechanism in Hepatocellular Carcinoma

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SUMMARY: The peroxisome proliferator-activated receptor-γ (PPARγ) high-affinity ligand, 15-deoxy-Δ-12,14-PGJ_2 (15d-PGJ_2), is toxic to malignant cells through cell cycle arrest and apoptosis induction. In this study, we investigated the effects of 15d-PGJ_2 on apoptosis induction and expression of apoptosis-related proteins in hepatocellular carcinoma (HCC) cells. 15d-PGJ_2 induced apoptosis in SK-Hep1 and HepG2 cells at a 50 μM concentration. Pretreatment with the pan-caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethyl ketone (2-VAD-fmk), only partially blocked apoptosis induced by 40 μM 15d-PGJ_2. This indicated that 15d-PGJ_2 induction of apoptosis was associated with a caspase-3–independent pathway. 15d-PGJ_2 also induced down-regulation of the *X* chromosome-linked inhibitor of apoptosis (XIAP), Bclx, and apoptotic protease-activating factor-1 in SK-Hep1 cells but not in HepG2 cells. However, 15d-PGJ_2 sensitized both HCC cell lines to TNF-related apoptosis-induced ligand–induced apoptosis. In SK-Hep1 cells, cell toxicity, nuclear factor-κB (NF-κB) suppression, and XIAP down-regulation were induced by 15d-PGJ_2 treatment under conditions in which PPARγ was down-regulated. These results suggest that the effect of 15d-PGJ_2 was through a PPARγ-independent mechanism. Although cell toxicity was induced when PPARγ was down-regulated in HepG2 cells, NF-κB suppression and XIAP down-regulation were not induced. In conclusion, 15d-PGJ_2 induces apoptosis of HCC cell lines via caspase-dependent and -independent pathways. In SK-Hep1 cells, the ability of 15d-PGJ_2 to induce cell toxicity, NF-κB suppression, or XIAP down-regulation seemed to occur via a PPARγ-independent mechanism, but in HepG2 cells, NF-κB suppression by 15d-PGJ_2 was dependent on PPARγ. (*Lab Invest 2003, 83:1529–1539*).

15-deoxy-Δ-12,14-PGJ_2 (15d-PGJ_2) is a prostaglandin J_2 (PGJ_2) derivative and is a high-affinity ligand selective for peroxisome proliferator-activated receptor-γ (PPARγ) (Forman et al, 1995). 15d-PGJ_2 activates PPARγ, which is functionally associated with adipocyte development (Forman et al, 1995), adipocyte differentiation (Kliewer et al, 1995), and inhibition of inducible nitric oxide synthesis in macrophages (Ricote et al, 1998). Through these physiologic actions, 15d-PGJ_2 contributes to the maintenance of tissue homeostasis.

Recently, it was reported that PPARγ is expressed in malignant cells and that ligand activation affects malignant cell proliferation and growth (Brockman et al, 1998; Chang and Szabo, 2000; Keelan et al, 1999; Motomura et al, 2000; Okano et al, 2002; Rumi et al, 2001; Sarraf et al, 1998; Tsubouchi et al, 2000). In malignant cells, activation of PPARγ induces cell cycle arrest (Brockman et al, 1998; Clay et al, 2001; Koga et al, 2001; Motomura et al, 2000; Rumi et al, 2001), cell differentiation (Chang and Szabo, 2000; Sarraf et al, 1998), or apoptosis (Keelan et al, 1999). These results imply that the PPARγ activation pathway may be a possible intervention mode for treatment of hepatocellular carcinomas (HCCs), which are resistant to current treatments.

PPARγ contributes to regulation of gene transcription in cells. In particular, activated PPARγ inhibits nuclear factor-κB (NF-κB) activity (Chinetti et al, 1998; Chung et al, 2000; Ji et al, 2001; Petrova et al, 1999; Ricote et al, 1998), which is associated with cell survival. In macrophages, PPARγ activation induces apoptosis by interfering with the antiapoptotic NF-κB signaling pathway (Chinetti et al, 1998; Ricote et al, 1998). NF-κB also regulates apoptosis-related gene expression and induces apoptosis-related protein expression in cells (Bui et al, 2001; Foehr et al, 2000; Kreuz et al, 2001; Micheau et al, 2001; Tamatani et al, 1999; Yabe et al, 2001; Yang et al, 2000), contributing to oncogenesis and tumor escape from immune surveillance (Dhanalakshmi et al, 2002; Hiscott et al, 2001; Javeland et al, 2002; Tan and Waldmann, 2002).

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The fact that the PPARγ ligand is a regulator of NF-κB activation implies an important association between cell apoptosis induction and PPARγ activation. However, few studies describe a relationship between PPARγ activation and apoptotic-related protein expression (Ohta et al, 2001), particularly in gastrointestinal malignant tumor cells (Toyoda et al, 2002).

In this study, we investigated the effect of 15d-PGJ_2 on induction of apoptosis and apoptosis-related proteins in human HCC cells. We examined potential mechanisms by which 15d-PGJ_2 induces apoptosis and increases expression of intracellular apoptosis-related proteins.

Results

The 15d-PGJ_2 PPARγ Ligand Induces HCC Cell Apoptosis

We previously showed that PPARγ is prevalent in human HCC cells (Okano et al, 2002). In the current study, we investigated the effect of 15d-PGJ_2 on the viability of SK-Hep1 and HepG2 HCC cells (Fig. 1a). As expected, 10 μM 15d-PGJ_2 failed to induce significant cytotoxicity in HCC cells after 24-hour incubation. However, 50 μM 15d-PGJ_2 did induce effective cell death in SK-Hep1 cells. HepG2 cell viability also was decreased by 50 μM 15d-PGJ_2, although its cytotoxic effect was less than that in SK-Hep1 cells.

We used 4'6-diamidino-2-phenylindole (DAPI) staining to evaluate whether HCC cells undergo apoptosis when treated with 50 μM 15d-PGJ_2. Untreated control cells did not show any typical apoptotic features (Fig. 1, b and d). In contrast, HCC cells treated with 50 μM 15d-PGJ_2 showed typical apoptotic features (Fig. 1, c and e). To verify cellular apoptosis, we used FITC-conjugated anti-annexin V antibody to evaluate the extent of phosphatidylserine translocation to the cell surface, such as would occur during apoptosis (Fig. 1, f to i). Untreated control cells did not show cell surface staining, whereas cells treated with 50 μM 15d-PGJ_2 showed cell surface staining of the anti-annexin V antibody, indicative of surface membrane phosphatidylserine expression.

15d-PGJ_2 Induces Apoptosis Via Caspase-3 and Caspase-3 Independent Pathways

PPARγ is a nuclear hormone receptor controlling gene transcription and regulating expression of cell cycle proteins in malignant cells through apoptosis induction (Koga et al, 2001; Motomura et al, 2000). It is reported that PPARγ activation can enhance apoptosis induced by TNF family receptor stimulation (Goke et al, 2000; Ji et al, 2001; Okano et al, 2002). Hence, we speculated that PPARγ activation might result in activation of the caspase cascade, and we examined

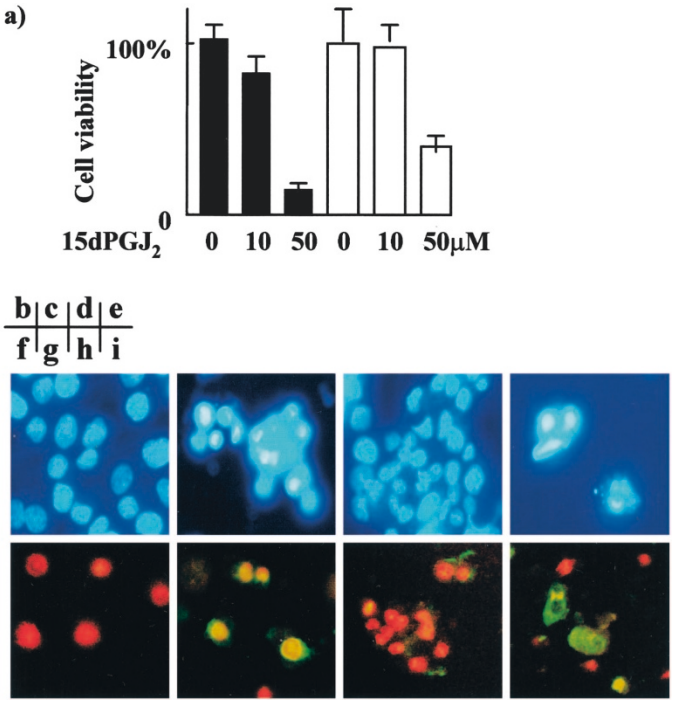

Figure 1.

a, Effect of 15-deoxy-Δ-12,14-PGJ_2 (15d-PGJ_2) stimulation on human hepatocellular carcinoma (HCC) cells (SK-Hep1: *solid bars*; HepG2: *open bars*). Cells were incubated with 15d-PGJ_2 for 24 hours at the indicated concentrations. Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Data shown are the mean ± SD of five independent experiments. b to e, 4'6-Diamidino-2-phenylindole (DAPI) staining was used to evaluate apoptosis in control, untreated SK-Hep1 (b) and HepG2 cells (d) as well as after 15d-PGJ_2 treatment (50 μM) of SK-Hep1 (c) and HepG2 (e) cells. Incubation of 50 μM 15d-PGJ_2 induced typical apoptosis features, including nuclear condensation and nuclear fragmentation. f to i, HCC cells were stained with FITC-conjugated anti-annexin V antibody and propidium iodide after incubation in the absence or presence of 50 μM 15d-PGJ_2 for 12 hours. Without 15d-PGJ_2, both SK-Hep1 (f) and HepG2 cells (h) showed only cell nuclei staining. After incubation with 15d-PGJ_2, cell surface staining with FITC-conjugated anti-annexin V antibody was observed on both SK-Hep1 (g) and HepG2 (i).

the expression of apoptosis-related proteins after 15d-PGJ$_2$ treatment. Caspase-3 expression was detected in both SK-Hep1 and HepG2 cells before treatment, and incubation with 50 μM 15d-PGJ$_2$ resulted in reduced caspase-3 protein expression in SK-Hep1 cells (Fig. 2a). Because the anti-caspase-3 antibody does not recognize cleaved caspase-3, the observed reduction is a result of cleavage and activation of caspase-3 after 15d-PGJ$_2$ treatment. In contrast, no change in caspase-3 expression was observed in HepG2 cells after 50 μM 15d-PGJ$_2$ treatment.

It was possible that a caspase-independent mechanism contributed to the apoptosis induced by 15d-PGJ$_2$ in HCC cells because HepG2 cells underwent apoptosis without apparent activation of caspase-3. Therefore, we investigated the effect of pretreatment with the pan-caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethyl ketone (Z-VAD-fmk), on 15d-PGJ$_2$-induced apoptosis. We observed that 40 μM Z-VAD-fmk partially blocked the ability of 15d-PGJ$_2$ to induce apoptosis in HCC cells (Fig. 2b), but this was incomplete and the inhibitor did not completely block the apoptosis.

15d-PGJ$_2$ Induces Down-Regulation of Other Apoptosis-Related Proteins

The previous data showed that apoptosis induction by 15d-PGJ$_2$ was partially associated with activation of caspase-3, the terminal enzyme of the caspase cascade, suggesting that PPARγ activation might modulate other apoptosis-related proteins. Expression of the *X* chromosome-linked inhibitor of apoptosis (XIAP), Bclx, apoptotic protease-activating factor-1 (Apaf-1), and FLICE/caspase-8-inhibitory protein (FLIP), were analyzed by Western blotting (Fig. 3). XIAP protein expression in HCC cells was evaluated because XIAP is a principal inhibitor of active caspase-3 in human HCC (Shiraki et al, 2002). We found that XIAP expression decreased in SK-Hep1 cells after 50 μM 15d-PGJ$_2$ incubation, whereas no change was observed in HepG2 cells (Fig. 3).

It is also known that XIAP interacts with processed caspase-9 and inhibits apoptosis (Silke et al, 2002). Caspase-9, which can activate several downstream caspases, including caspase-3, is induced by autoactivation via the Apaf-1/cytochrome c complex. Cytochrome c is released from mitochondria, and Bcl-2 family members, including Bclx, are principal regulators of the mitochondria-initiated caspase activation pathway (Shiraki et al, 2002). Hence, we examined expression of Bclx and Apaf-1 after treatment of HCC cells with15d-PGJ$_2$ (Fig. 3). Expression of Bclx was slightly down-regulated by 50 μM 15d-PGJ$_2$ in SK-Hep1 cells, with no change in expression in HepG2 cells. Apaf-1 also was down-regulated by 50 μM 15d-PGJ$_2$ in SK-Hep1 cells, and a 17-kDa fragment was observed, probably as a result of degradation by caspase-3 (Lauber et al, 2001). However, Apaf-1 was

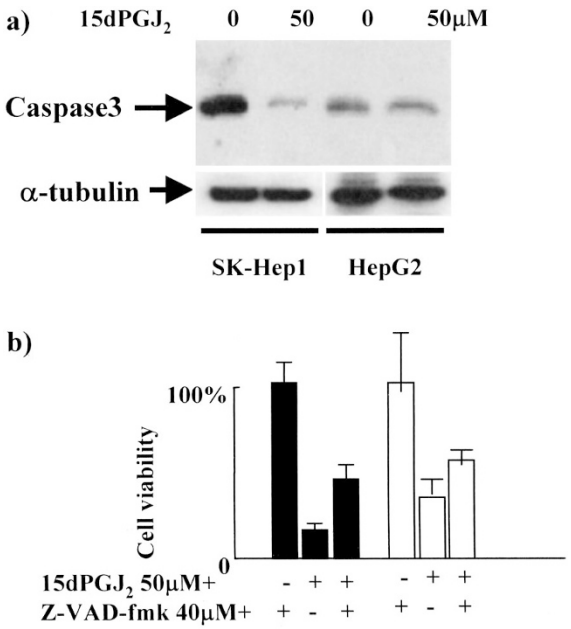

Figure 2.

a, Expression of caspase-3 determined by Western blotting. SK-Hep1 and HepG2 cells were incubated in the presence or absence of 50 μM 15d-PGJ$_2$ for 24 hours at 37° C. *Arrows* indicate the expression of caspase-3 (32 kDa). b, Restoration of cell viability by benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethyl ketone (Z-VAD-fmk) pretreatment of HCC cells (SK-Hep1, *solid bars*; HepG2, *open bars*). Z-VAD-fmk pretreatment followed by 15d-PGJ$_2$ incubation partially restored cell viabilities as compared with 15d-PGJ$_2$ alone.

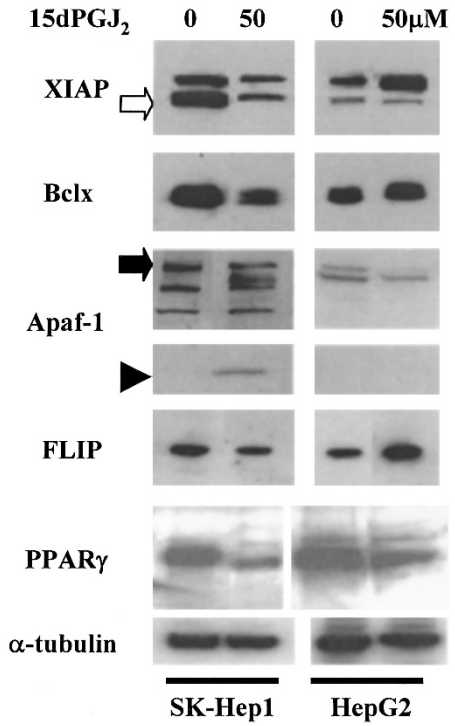

Figure 3.

Expression of the *X* chromosome-linked inhibitor of apoptosis (XIAP), Bclx, apoptotic protease-activating factor-1 (Apaf-1), FLICE/caspase-8-inhibitory protein (FLIP), or proliferator-activated receptor-γ (PPARγ) determined by Western blotting. SK-Hep1 and HepG2 cells were incubated in the presence or absence of 50 μM 15d-PGJ$_2$ for 24 hours at 37° C. *Open arrow* indicates XIAP, *solid arrow* indicates uncleaved Apaf-1 (130 kDa), and *solid arrowhead* indicates cleaved Apaf-1 (17 kDa) fragment.

not down-regulated in treated HepG2 cells, nor was the 17-kDa fragment observed.

FLIP, which acts near the beginning of the caspase cascade and inhibits formation of the death-inducing signal complex (Shiraki et al, 2002), slightly decreased in SK-Hep1 cells, but increased in HepG2 cells after 15d-PGJ_2 treatment. The expression of PPARγ was down-regulated in both HCC cell lines (Fig. 3).

15d-PGJ_2 Enhances TNF-related apoptosis-induced ligand (TRAIL)–Induced Apoptosis in HCC Cells

As shown above, 15d-PGJ_2 seemed to influence apoptosis-related protein expression in HCC cells. Bclx and Apaf-1 are associated with apoptosis induction via a mitochondrial pathway (Tsujimoto and Shimizu, 2000; Zou et al, 1997), and a recent study revealed that the mitochondrial pathway is an important determinant for apoptosis of tumor cells by TRAIL (Thomas et al, 2000). Therefore, we examined the effect of 15d-PGJ_2 on TRAIL-induced apoptosis of HCC cells (Fig. 4). TRAIL treatment alone (100 ng/ml) had a minimal effect on SK-Hep1 and HepG2 cells. In contrast, both HCC cell lines were effectively killed with 50 μM 15d-PGJ_2, and the rate of cell death was enhanced by costimulation with TRAIL and 15d-PGJ_2.

15d-PGJ_2 Suppressed NF-κB Activation and Induced Down-Regulation of XIAP Expression in HCC Cells

Because PPARγ activation inhibits NF-κB activity (Chinetti et al, 1998; Chung et al, 2000; Ji et al, 2001; Petrova et al, 1999; Ricote et al, 1998), we examined the effect of 15d-PGJ_2 on NF-κB activation in HCC cells. In SK-Hep1 and HepG2 cells, treatment with 50 ng/ml TNF-α effectively induced NF-κB activation after 8-hour incubation and 15d-PGJ_2 treatment attenuated its activation (Fig. 5). This suppression of NF-κB activation by 15d-PGJ_2 was greater in SK-Hep1 cells than in HepG2 cells.

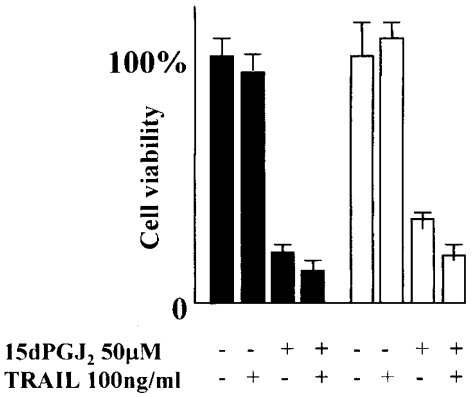

Figure 4.

Effects of 15dPGJ_2 and TNF-related apoptosis-induced ligand (TRAIL) on human HCC cell viability (SK-Hep1, *solid bars*; HepG2, *open bars*). HCC cells were incubated with 100 ng/ml TRAIL, 50 μM 15d-PGJ_2, or with a mixture of TRAIL and 15d-PGJ_2 for 24 hours. Cell viability was assessed by MTT assay. The data shown are the mean $\pm$ SD of five independent experiments. The combination of TRAIL and 15dPGJ_2 treatment promoted cell death compared with each individual treatment.

Since there was no change in XIAP expression in HepG2 cells (Fig. 3), it was possible that insufficient inhibition of NF-κB activation might be a contributing factor. To further analyze XIAP regulation, we transfected HCC cells with an IκBα expression vector and the cells were tested for inhibition of NF-κB activation (Fig. 5b). NF-κB activation was suppressed in the transfected cells, and there was reduced XIAP expression in both SK-Hep1 cells and HepG2 cells. There was no corresponding change in Bclx or caspase-3 expression, suggesting that inhibition of NF-κB activation by overexpressing IκBα selectively affected XIAP down-regulation in HCC cells.

15d-PGJ_2 Effects on HCC Viability Are Independent of PPARγ Pathways

Previously, 15d-PGJ_2 was thought to exert its effects on cells exclusively through PPARγ; however, recent reports describe PPARγ-independent mechanisms (Harris et al, 2002; Petrova et al, 1999; Rossi et al, 2000; Thieringer et al, 2000; Vaidya et al, 1999). It is not known whether PPARγ-independent signaling pathways exist in gastrointestinal malignant tumor cells. Therefore, we induced down-regulation of PPARγ in HCC cells using PPARγ antisense oligodeoxynucleotides. HCC cells were incubated with 1 μM PPARγ antisense or sense oligodeoxynucleotides, and expression of PPARγ was analyzed by Western blotting. HCC cells transfected with PPARγ antisense oligos showed reduced PPARγ expression (Fig. 6a), compared with cells that received sense oligos. In cells transfected with sense oligos, 15d-PGJ_2 induced cell death (Fig. 6b). This loss of viability was even greater in HCC cells treated with PPARγ antisense oligos for both SK-Hep1 cells and HepG2 cells. Collectively, these results suggested that 15d-PGJ_2 affected PPARγ-independent pathways that contribute to HCC cell death.

15d-PGJ_2 Regulates NF-κB Activation Through PPARγ-Dependent and -Independent Pathways

To further evaluate the ability of 15d-PGJ_2 to influence PPARγ-independent pathways, we investigated NF-κB activation by 15d-PGJ_2 when PPARγ was down-regulated. In SK-Hep1 cells, 15d-PGJ_2 inhibited NF-κB activation induced by TNF-α when PPARγ was normally expressed (*open bars*) or down-regulated (*solid bars*). However, in HepG2 cells, down-regulation of PPARγ interfered with 15d-PGJ_2 effects such that TNF-α induced NF-κB activation even in the presence of 15d-PGJ_2 (Fig. 7a). We evaluated expression of apoptosis-related proteins by 15d-PGJ_2 treatment when PPARγ expression was down-regulated (Fig. 7b). In SK-Hep1 cells, 15d-PGJ_2 reduced XIAP expression regardless of the PPARγ expression level. Caspase-3 also was down-regulated, but to a greater extent in cells expressing less PPARγ. There were no changes in XIAP expression after 15d-PGJ_2 treatment of HepG2 cells (Fig. 7b). 15d-PGJ_2 treatment induced a slight up-regulation of caspase-3 with PPARγ down-

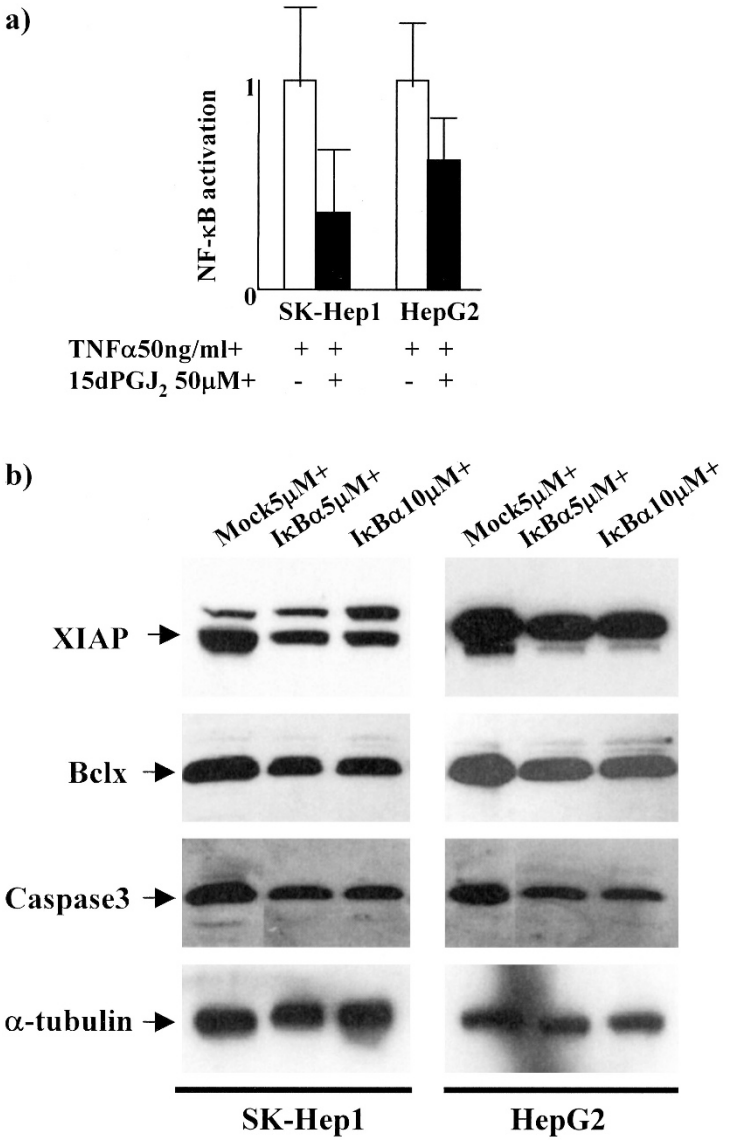

Figure 5.

a, Effects of 15d-PGJ$_2$ on NF-κB activation after TNF-α treatment of HCC cells. Cells were incubated with 50 ng/ml TNF-α alone (*open bars*) or pretreated for 24 hours with 50 ng/ml TNF-α followed by 50 μM 15d-PGJ$_2$ (*solid bars*). Reporter gene activity was measured 6 hours after treatment. NF-κB activation was assessed by a luciferase assay. Data shown are the mean ± SD of three independent experiments. b, Expression of apoptotic-related proteins (XIAP and caspase-3) after transient transfection with IκBα expression vector or control vector. Protein expression was analyzed by Western blotting. The IκBα vector transfection induced a decrease in XIAP expression levels but did not affect caspase-3.

regulation, compared with cells with normal expression of PPARγ. Thus, it seemed that 15d-PGJ$_2$ suppressed NF-κB activation through PPARγ-dependent and -independent mechanisms and regulated XIAP expression in HCC cells (Fig. 8).

Discussion

In this study we demonstrated that 15d-PGJ$_2$, a natural cyclopentenone prostaglandin and PPARγ agonist, induced apoptosis in HCC cells. This occurred with variable caspase-3 activation; pretreatment with Z-VAD-fmk, a pan-caspase inhibitor, only partially interfered with the 15d-PGJ$_2$ induction of apoptosis in HCC cells. This suggests that 15d-PGJ$_2$ induces apoptosis using both caspase-dependent and -independent pathways in HCC cells. Some reports demonstrate caspase-3-independent apoptotic pathways, including apoptosis induced by exogenous nitric oxide, transforming growth factor-β, cell-permeable peptide SN50, arsenic trioxide (As_2O_3), NF-κB inhibition, or a low-molecular weight fraction of human seminal plasma (Brown et al, 1999; Kolenko et al, 1999; Mohr et al, 1998; Pagliari et al, 2000; Perfettini et al, 2002; Sternsdorf et al, 1999; Untergasser et al, 2001). It is also reported that a caspase-independent mechanism is partly involved in the 15d-PGJ$_2$–induced apoptosis of malignant cells (Nishida et al, 2002), which is consistent with the current findings.

During caspase-3–independent apoptosis, several studies show mitochondrial potential changes and

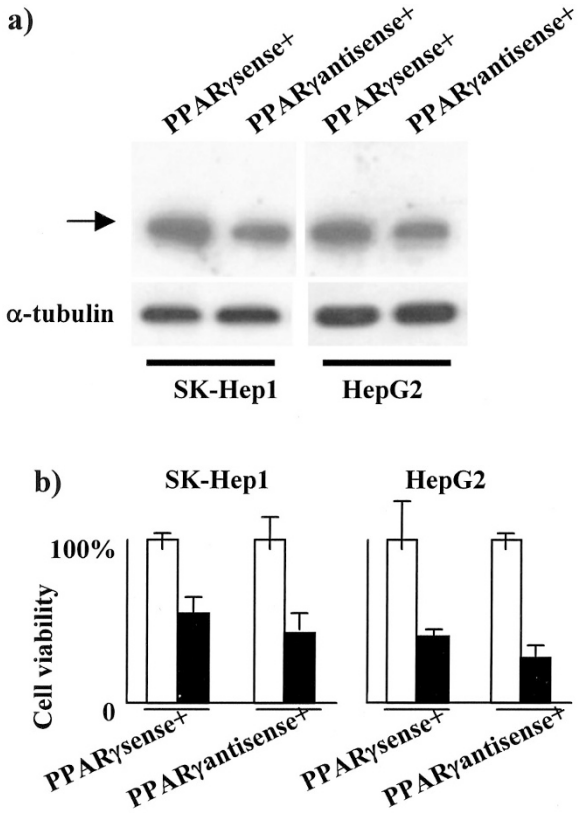

Figure 6.

a, Down-regulation of PPARγ expression in HCC cells (SK-Hep1 and HepG2) after transfection with PPARγ antisense oligos. HCC cells were transfected with 1 μM PPARγ sense (control) or antisense oligodeoxynucleotides using Fugene 6 and incubated for 24 hours at 37° C. PPARγ expression decreased after transfection with the antisense oligos. b, Cell viability after down-regulation of PPARγ expression. HCC cells were incubated in the absence (*open bars*) or presence (*solid bars*) of 50 μM 15d-PGJ_2 for 24 hours. Cell viability was assessed by MTT assay. The data shown are the mean ± SD of five independent experiments.

changes in expression of antiapoptotic proteins (Pagliari et al, 2000; Untergasser et al, 2001). Our study also demonstrated down-regulation of XIAP, Bclx, and Apaf-1 by 15d-PGJ_2 in SK-Hep1 cells. In contrast, FLIP was not down-regulated in SK-Hep1 and HepG2 cells, and no changes in apoptosis-related proteins were observed in HepG2 cells. Because degradation of FLIP by PPARγ ligand is possible (Kim et al, 2002), the differences we observe may be characteristic of each HCC cell line. Down-regulation of these antiapoptotic proteins may promote TRAIL-induced apoptosis in HCC cells. Because 15d-PGJ_2 treatment enhanced cell death induced by TRAIL in HepG2 cells, other apoptosis-related proteins may be affected by 15d-PGJ_2, such as Bcl-2, Bax, inhibitor of apoptosis-1, or inhibitor of apoptosis-2. The functional basis that links expression of these apoptosis-related proteins and 15d-PGJ_2 treatment should be assessed to more fully understand PPARγ-induced apoptosis of malignant cells.

NF-κB activation induces specific gene expression that tightly regulates programmed cell death and inhibition of apoptosis (Barkett and Gilmore, 1999; Beg et al, 1995; Schmid and Adler, 2000). In agreement with previous reports (Chinetti et al, 1998; Chung et al, 2000; Ji et al, 2001; Petrova et al, 1999; Ricote et al, 1998), our data demonstrated that 15d-PGJ_2 inhibited NF-κB activation, particularly in SK-Hep1 cells. However, inhibition of NF-κB activation by 15d-PGJ_2 was weak in HepG2 cells. Because overexpression of IκBα induced down-regulation of XIAP expression in both SK-Hep1 and HepG2 cells, XIAP down-regulation by 15d-PGJ_2 in HCC cells may be regulated through NF-κB activation and thus the effect of 15d-PGJ_2 on NF-κB activation may be weak. On the other hand, Bclx was not down-regulated by overexpression of IκBα. PPARγ activation inhibits NF-κB activation, as well as phosphatidylinositol-3-kinase/Akt (Goetze et al, 2002), activator protein-1, and signal transducers and activators of transcription (Ricote et al, 1998). Inhibition of these pathways by 15d-PGJ_2 may also affect expression of apoptosis-related proteins in HCC cells.

Although 15d-PGJ_2 is a high-affinity ligand for PPARγ and is associated with cell death and gene expression through PPARγ activation, 15d-PGJ_2 also has PPARγ-independent signaling pathways. These independent mechanisms include suppression of inducible nitric oxide synthase activity (Hortelano et al, 2000; Petrova et al, 1999), modulation of reactive oxygen intermediates production (Vaidya et al, 1999), induction of IL-8 (Harris et al, 2002), stimulation of apoptosis of hepatic myofibroblasts (Li et al, 2001), and inhibition of NF-κB and activator protein-1 activation (Boyault et al, 2001). In this study, we demonstrated that 15d-PGJ_2 was toxic to HCC cells and suppressed NF-κB activation through a PPARγ-independent pathway in SK-Hep1 cells. However, in HepG2 cells, suppression of NF-κB activation was not observed in cells with low PPARγ expression, although apoptosis was induced. This suggests that apoptosis by 15d-PGJ_2 is induced mainly via a PPARγ-independent mechanism in HCC cells. The effect of 15d-PGJ_2 on NF-κB activation by may be influenced by dual, overlapping pathways that may or may not involve PPARγ. In SK-Hep1 cells, 15d-PGJ_2 may inhibit NF-κB activation via primarily a PPARγ-independent mechanism, whereas in HepG2 cells, a PPARγ-dependent mechanism may predominate.

In SK-Hep1 cells, reduced XIAP expression was observed in cells with normal and down-regulated PPARγ expression levels after suppression of NF-κB by 15d-PGJ_2. This result supports the notion that 15d-PGJ_2 regulates XIAP expression via a PPARγ-independent mechanism in SK-Hep1 cells. Because the ability of 15d-PGJ_2 to inhibit NF-κB activation was relatively weak in HepG2 cells (Fig. 5a), XIAP expression may not be regulated by 15d-PGJ_2, irrespective of PPARγ expression in HepG2 cells. XIAP down-regulation, which sensitizes tumor cells to TRAIL-induced apoptosis, may be an alternative treatment pathway, possible via TNF-α or Fas.

In conclusion, 15d-PGJ_2 induces apoptosis in HCC cells and inhibits NF-κB activation and XIAP expression via a PPARγ-independent mechanism. There are malignant cells with minimal or no PPARγ expression (Elstner et al, 1998; Ohta et al, 2001), suggesting that cell toxicity

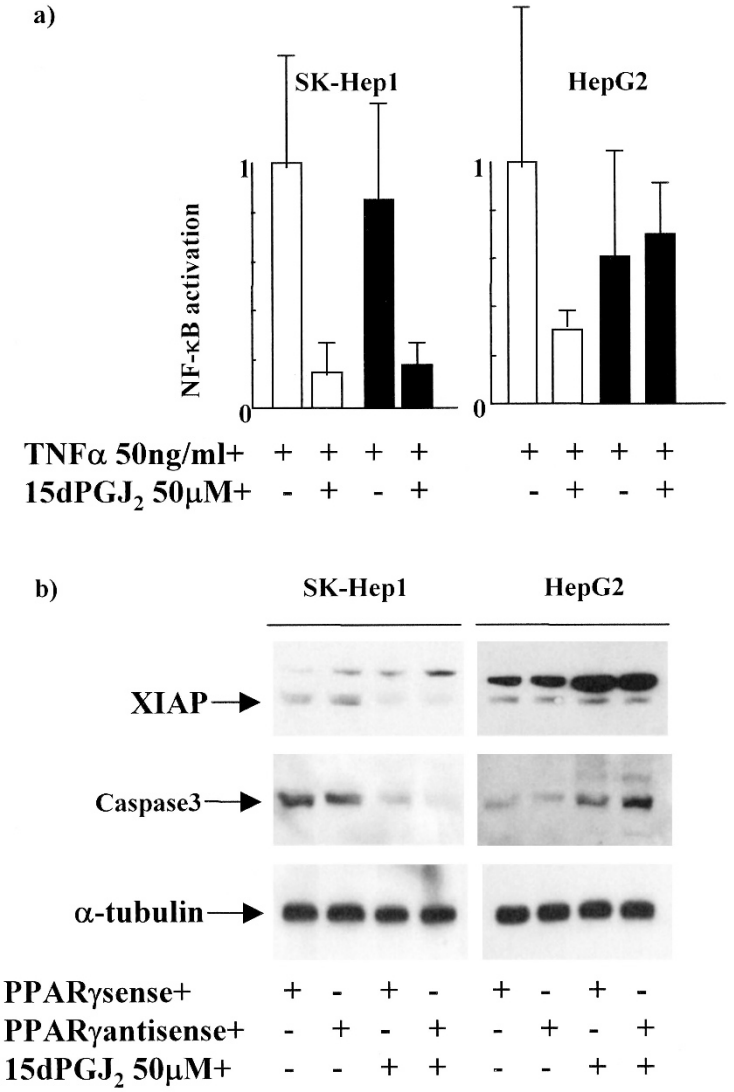

Figure 7.

a, The effects of down-regulated PPARγ expression on NF-κB activation in HCC cells. Cells were transfected with 1 μM PPARγ sense oligodeoxynucleotides (*open bars*) or 1 μM PPARγ antisense oligodeoxynucleotides (*solid bars*). HCC cells were pretreated with 50 ng/ml TNF-α for 2 hours and then incubated in the absence or presence with 50 μM 15d-PGJ$_2$ for 6 hours. NF-κB activation was assessed by a luciferase assay. Data shown are the mean $\pm$ SD of three independent experiments. In SK-Hep1 cells, down-regulation of PPARγ expression did not change NF-κB activation levels. In HepG2 cells, the down-regulation of PPARγ expression restored NF-κB activation levels. b, The expression of XIAP and caspase-3 after down-regulation of PPARγ expression. HCC cells were transfected with PPARγ sense or antisense oligos and incubated in the absence or presence of 50 μM 15d-PGJ$_2$ for 24 hours. Protein expression was analyzed by Western blotting.

pathways independent of PPARγ should be further investigated for induction of tumor cell apoptosis. Down-regulation of apoptosis inhibitory proteins by 15d-PGJ$_2$ may increase the sensitivity of tumor cells to TRAIL and sensitize them to TNF-family receptor signaling, opening up new opportunities for therapeutic intervention.

Materials and Methods

Cell Lines and Reagents

The HCC cell lines, HepG2 and SK-Hep1 cells, were purchased from the American Type Culture Collection (Rockville, Maryland). HLE (JCRB 0404) was purchased from the Health Science Research Resource Bank (Osaka, Japan). These cell lines were cultured in DMEM (Dainippon Pharmaceutical Company, Ltd., Osaka, Japan) at 37° C. All media were supplemented with 1% penicillin/streptomycin (GIBCO BRL, Grand Island, New York) and 10% heat-inactivated FCS (GIBCO BRL). 15d-PGJ$_2$ was purchased from Cayman Chemicals (Ann Arbor, Michigan). Anti-Caspase-3, Bclx, and Apaf-1 antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, California). Anti-FLIP antibody was purchased from Millennium Biotechnology (Romona, California). Anti-XIAP antibody was purchased from BD Bioscience (Franklin Lakes, New Jersey). Anti-PPARγ1,2 polyclonal antibody was purchased from CALBIOCHEM (San Diego, California).

Assessment of HCC Cell Viability

To assess HCC cell viability, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was

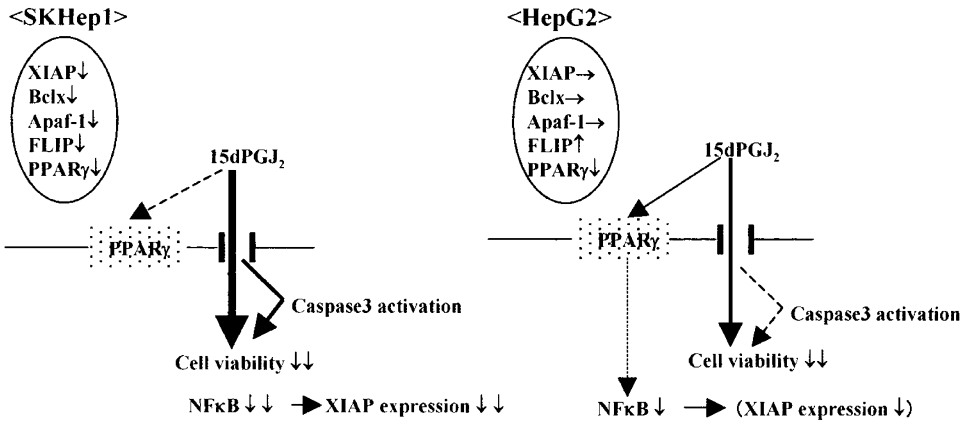

Figure 8.

A schematic outline of the proposed mechanism of 15d-PGJ_2 signaling pathways in SK-Hep1 and HepG2 HCC cell lines. Cell toxicity with 15d-PGJ_2 is exerted through a PPARγ-independent pathway in both SK-Hep1 and HepG2 cells. Cell toxicity with 15d-PGJ_2 partially accompanies caspase-3 activation in SK-Hep1 cells. In HepG2 cells, 15d-PGJ_2 weakly activates caspase-3. NF-κB activation is differentially suppressed in SK-Hep1 and HepG2 cells. In SK-Hep1 cells, 15d-PGJ_2 suppresses NF-κB activation through a PPARγ-independent pathway, whereas in HepG2 cells the signaling pathway of NF-κB suppression by 15d-PGJ_2 is via PPARγ. The suppression of NF-κB activation results in XIAP down-regulation in both HCC cell lines.

performed. The HCC cells were plated at a density of 5 $\times$ 10^3 cells per well in 96-well microtiter plates (Corning Glass Works, Corning, New York), and each plate was incubated for 24 hours at 37° C in 5% CO_2. Each reagent was added, and the plate was incubated for 24 hours. The live-cell count was determined using a Cell Titer 96 assay kit (Promega, Madison, Wisconsin) according to the manufacturer's instructions. The absorbance of each well was measured at 570 nm with a microtiter plate reader (Bio-Rad Laboratories, Hercules, California).

Detection of Apoptosis

A total of 2 $\times$ 10^4 HCC cells per well was cultured in an 8-well Lab-tek II chamber slide (Nalge Nunc International, Rochester, New York) for 24 hours, followed by addition of 50 μM 15d-PGJ_2 (Cayman Chemicals). After incubation for 24 hours, cell nuclei were stained with DAPI (Sigma, St. Louis, Missouri) and observed with a fluorescence microscope (Zeiss, Göttingen, Germany). To detect early apoptotic changes, cells were incubated with 50 μM 15d-PGJ_2 for 12 hours, and expression of cell surface phosphatidylserine was determined with an Annexin V-FITC apoptosis detection kit (MBL Company, Ltd., Nagoya, Japan).

Western Blotting Analysis of HCC Cell Extracts After 15d-PGJ_2 Stimulation

HCC cells (4 $\times$ 10^5; SK-Hep1, HLE, or HepG2) were grown in 60-mm dishes for 24 hours at 37° C in 5% CO_2 the day before reagent addition. HCC cells were incubated with 0, 10, or 50 μM 15d-PGJ_2 (Caymen Chemicals) for 24 hours at 37° C in 5% CO_2. After incubation, HCC cells were harvested and lysed in lysis buffer (50 mmol/L Tris-HCl, pH 8, 150 mmol/L NaCl, 5 mmol/L ethylenediaminetetraacetic acid, 1% NP-40, 1 mmol/L phenylmethylsulfonyl fluoride) on ice. After centrifugation, supernatants were collected and their protein content was measured using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories). Equal amounts of protein from each extract were separated by 14% SDS-PAGE and transferred onto nitrocellulose membranes (Toyo Roshi, Tokyo, Japan) using the Bio-Rad electrotransfer system. Blots were blocked by incubation in 5% nonfat dried milk in Tris-buffered saline overnight at 4° C and probed for 2 hours at room temperature with each antibody. Antibodies were diluted 1:1000 with 0.05% Tween 20 in Tris-buffered saline. The immunoblots were then probed with horseradish peroxidase-conjugated anti-rabbit IgG (Amersham Biosciences Corp., Piscataway, New Jersey), anti-mouse IgG (Santa Cruz Biotechnology), or anti-goat IgG (Zymed Laboratory Inc., South San Francisco, California) at 1:2000 dilutions in 5% nonfat dried milk in Tris-HCl, pH 7.5, and 0.05% Tween 20. After the final washing, signal was detected with an ECL kit (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom).

NF-κB Luciferase Reporter Gene Assay

The pNF-κB-Luc Vector (Mercury Pathway Profiling System) and pCMV-IκBα were obtained from Clontech (San Diego, California). Human HCC cells (2 $\times$ 10^5) were grown in 6-well plates (NUNC Brand Products, Demmark) the day before transfection. Cells were transfected using FuGENE 6 (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's protocol. HCC cells were pretreated with 50 ng/ml TNF-α for 2 hours before treatment with 50 μM 15d-PGJ_2. Reporter gene activity was measured 6 hours after 15d-PGJ_2 treatment.

PPARγ Antisense Oligodeoxynucleotide Transfection in HCC Cells

To inhibit PPARγ protein expression in HCC cells, phosphorothiorate antisense oligodeoxynucleotides were used to inhibit the FLIP initiation codon. Control cells were transfected with sense oligodeoxynucleoti-

des. The following sequences were used (Nikitakis et al, 2002): PPARγ antisense, 5'-ctctgtgtcaaccatggtca-3'; PPARγ sense, 5'-atgaccatggttgacacaga-3'. A total of 5×10^5 HCC cells per well was transfected with 1 μM PPARγ antisense or sense oligodeoxynucleotides using FuGENE 6 (Boehringer Mannheim) according to the manufacturer's protocol and incubated for 24 hours at 37° C. PPARγ expression was analyzed by Western blotting.

References

Barkett M and Gilmore T (1999). Control of apoptosis by Rel/NF-κB transcription factors. Oncogene 18:6910–6924.

Beg AA, Sha WC, Bronson RT, Ghosh S, and Baltimore D (1995). Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-κB. Nature 376:167–170.

Boyault S, Simonin MA, Bianchi A, Compe E, Liagre B, Mainard D, Becuwe P, Dauca M, Netter P, Terlain B, and Bordji K (2001). 15-Deoxy-$\Delta^{12,14}$-PJ$_2$, but not troglitazone, modulates IL-1β effects in human chondrocytes by inhibiting NF-κB and AP-1 activation pathways. FEBS Lett 501:24–30.

Brockman JA, Gupta RA, and DuBois RN (1998). Activation of PPARγ leads to inhibition of anchorage-independent growth of human colorectal cancer cells. Gastroenterology 115:1049–1055.

Brown TL, Patil S, Cianci CD, Morrow JS, and Howe PH (1999). Transforming growth factor β induces caspase 3-independent cleavage of αII-spectrin (α-fodrin) coincident with apoptosis. J Biol Chem 274:23256–23262.

Bui NT, Livolsi A, Peyron JF, and Prehn JHM (2001). Activation of nuclear factor κB and *bcl-x* survival gene expression by nerve growth factor requires tyrosine phosphorylation of IκBα. J Cell Biol 152:753–763.

Chang TH and Szabo E (2000). Induction of differentiation and apoptosis by ligands of peroxisome proliferator-activated receptor γ in non-small cell lung cancer. Cancer Res 60:1129–1138.

Chinetti G, Griglio S, Antonucci M, Torra IP, Delerive P, Majd Z, Fruchart JC, Chapman J, Najib J, and Staels B (1998). Activation of proliferator-activated receptors α and γ induces apoptosis of human monocyte-derived macrophages. J Biol Chem 273:25573–25580.

Chung SW, Kang BY, Kim SH, Pak YK, Cho D, Trinchieri G, and Kim TS (2000). Oxidized low density lipoprotein inhibits interleukin-12 production in lipopolysaccharide-activated mouse macrophages via direct interactions between peroxisome proliferator-activated receptor-γ and nuclear factor-κB. J Biol Chem 275:32681–32687.

Clay CE, Atsumi G, High KP, and Chilton FH (2001). Early de novo gene expression is required for 15-deoxy-Δ-12,14-prostaglandin J$_2$-induced apoptosis in breast cancer cells. J Biol Chem 276:47131–47135.

Dhanalakshmi S, Singh RP, Agarwal C, and Agarwal R (2002). Silibinin inhibits constitutive and TNFalpha-induced activation of NF-kappaB and sensitizes human prostate carcinoma DU145 cells to TNFalpha-induced apoptosis. Oncogene 21: 1759–1767.

Elstner E, Müller C, Koshizuka K, Williamson EA, Park D, Asou H, Shintaku P, Said JW, Heber D, and Koeffler HP (1998). Ligands for peroxisome proliferator-activated receptor γ and retinoic acid receptor inhibit growth and induce apoptosis of human breast cancer cells in vitro and in BNX mice. Proc Natl Acad Sci USA 95:8806–8811.

Foehr ED, Lin X, O'Mahony A, Geleziunas R, Bradshaw RA, and Greene WC (2000). NF-κB signaling promotes both cell survival and neurite process formation in nerve growth factor-stimulated PC12 cells. J Neurosci 20:7556–7563.

Forman BM, Tontonoz P, Chen J, Burn RP, Spiegelman BM, and Evans RM (1995). 15-Deoxy-delta 12,14-prostaglandin J2 is a ligand for the adipocyte determination factor PPARγ. Cell 83:803–812.

Goetze S, Eilers F, Bungenstock A, Kintscher U, Stawowy P, Blaschke F, Graf K, Law RE, Fleck E, and Grafe M (2002). PPAR activators inhibit endothelial cell migration by targeting Akt. Biochem Biophys Res Commun 293:1431–1437.

Goke R, Goke A, Goke B, and Chen Y (2000). Regulation of TRAIL-induced apoptosis by transcription factors. Cell Immunol 201:77–82.

Harris SG, Smith RS, and Phipps RP (2002). 15-deoxy-Δ-12,14-PGJ$_2$ induces IL-8 production in human T cells by a mitogen-activated protein kinase pathway. J Immunol 168: 1372–1379.

Hiscott J, Kwon H, and Génin P (2001). Hostile takeover: Viral appropriation of the NF-κB pathway. J Clin Invest 107:143–151.

Hortelano S, Castrillo A, Alvarez AM, and Boscá L (2000). Contribution of cyclopentenone prostaglandins to the resolution of inflammation through the potentiation of apoptosis in activated macrophages. J Immunol 165:6525–6531.

Javeland D, Poupon MF, Wietzerbin J, and Besancon F (2002). Inhibition of constitutive NF-kappaB activity suppresses tumorigenicity of Ewing sarcoma EW7 cells. Int J Cancer 98:193–198.

Ji JD, Cheon H, Jun JB, Choi SJ, Kim YR, Lee YH, Kim TH, Chae IJ, Song GG, Yoo DH, Kim SY, and Sohn J (2001). Effects of peroxisome proliferator-activated receptor-γ (PPARγ) on the expression of inflammatory cytokines and apoptosis induction in rheumatoid synovial fibroblast and monocytes. J Autoimmun 17:215–221.

Keelan JA, Sato TA, Marvin KW, Lander J, Gilmour RS, and Mitchell MD (1999). 15-Deoxy-Δ-12,14-prostaglandin J$_2$, a ligand for peroxisome proliferator-activated receptor-γ, induces apoptosis in JEG3 choriocarcinoma cells. Biochem Biophys Res Commun 262:579–585.

Kim Y, Suh N, Sporn M, and Reed JC (2002). An inducible pathway for degradation of FLIP protein sensitizes tumor cells to TRAIL-induced apoptosis. J Biol Chem 277:22320–22329.

Kliewer SA, Lenhard JM, Willson TM, Patel I, Morris DC, and Lehmann JM (1995). A prostaglandin J2 metabolite binds peroxisome proliferator-activated receptor gamma and promotes adipocyte differentiation. Cell 83:813–819.

Koga H, Sakisaka S, Harada M, Takagi T, Hanada S, Taniguchi E, Kawaguchi T, Sasatomi K, Kimura R, Hashimoto O, Ueno T, Yano H, Kojiro M, and Sata M (2001). Involvement of p21$^{WAF/Cip1}$, p27^{Kip1}, and p18^{INK4c} in troglitazone-induced cell-cycle arrest in human hepatoma cell lines. Hepatology 33:1087–1097.

Kolenko V, Bloom T, Rayman P, Bukowski R, His E, and Finke J (1999). Inhibition of NF-κB activity in human T lymphocytes induces caspase-dependent apoptosis without

detectable activation of caspase-1 and -3. J Immunol 163: 590–598.

Kreuz S, Siegmund D, Scheurich P, and Wajant H (2001). NF-κB inducers upregulate cFLIP, a cycloheximide-sensitive inhibitor of death receptor signaling. Mol Cell Biol 21:3964–3973.

Lauber K, Appel HAE, Schlosser SF, Gregor M, Schulze-Osthoff K, and Wesselborg S (2001). The adapter protein apoptotic protease-activating factor-1 (Apaf-1) is proteolytically processed during apoptosis. J Biol Chem 276:29772–29781.

Li L, Tao J, Davaille J, Feral C, Mallat A, Rieusset J, Vidal H, and Lotersztajn S (2001). 15-Deoxy-$\Delta^{12,14}$-prostaglandin J2 induces apoptosis of human hepatic myofibroblasts. J Biol Chem 276:38152–38158.

Micheau O, Lens S, Gaide O, Alevizopoulos K, and Tschopp J (2001). NF-κB signals induce the expression of c-FLIP. Mol Cell Biol 21:5299–5305.

Mohr S, McCormick TS, and Lapetina EG (1998). Macrophages resistant to endogenously generated nitric oxide-mediated apoptosis are hypersensitive to exogenously added nitric oxide donors: Dichotomous apoptotic response independent of caspase 3 and reversal by the mitogen-activated protein kinase kinase (MEK) inhibitor PD 098059. Proc Natl Acad Sci USA 95:5045–5050.

Motomura W, Okumura T, Takahashi N, Obara T, and Kohgo Y (2000). Activation of peroxisome proliferator-activated receptor γ by troglitazone inhibits cell growth through the increase of $p27^{kip1}$ in human pancreatic carcinoma cells. Cancer Res 60:5558–5564.

Nikitakis NG, Hamburger AW, and Sauk JJ (2002). The nonsteroidal anti-inflammatory drug sulindac causes down-regulation of signal transduction and activator of transcription 3 in human oral squamous cell carcinoma cells. Cancer Res 62:1004–1007.

Nishida K, Furumatsu T, Takada I, Kawai A, Yoshida A, Kunisada T, and Inoue H (2002). Inhibition of human chondrosarcoma cell growth via apoptosis by peroxisome proliferator-activated receptor-γ. Br J Cancer 86:1303–1309.

Ohta K, Endo T, Haraguchi K, Hershman JM, and Onaya T (2001). Ligands for peroxisome proliferator-activated receptor γ inhibit growth and induce apoptosis of human papillary thyroid carcinoma cells. J Clin Endocrinol Metab 86:2170–2177.

Okano H, Shiraki K, Inoue H, Yamanaka T, Deguchi M, Sugimoto K, Sakai T, Ohmori S, Fujikawa K, Murata K, and Nakano T (2002). Peroxisome proliferator-activated receptor γ (PPARγ) augments tumor necrosis factor (TNF) family-induced apoptosis in hepatocellular carcinoma. Anticancer Drugs 13:59–65.

Pagliari LJ, Perlman H, Liu H, and Pope RM (2000). Macrophages require constitutive NF-κB activation to maintain A1 expression and mitochondrial homeostasis. Mol Cell Biol 20:8855–8865.

Perfettini JL, Reed JC, Israel N, Martinou JC, Dautry-Varsat A, and Ojcius DM (2002). Role of Bcl-2 family members in caspase-independent apoptosis during *Chlamydia* infection. Infect Immun 70:55–61.

Petrova TV, Akama KT, and Van Eldik LJ (1999). Cyclopentenone prostaglandins suppress activation of microglia: Down-regulation of inducible nitric-oxide synthase by 15-Deoxy-Δ-12,14-prostaglandin J_2. Proc Natl Acad Sci USA 96:4668–4673.

Ricote M, Li AC, Willsons TM, Kelly CJ, and Glass CK (1998). The peroxisome proliferator-activated receptor-γ is a negative regulator of macrophage activation. Nature 391:79–82.

Rossi A, Kapahi P, Natoli G, Takahashi T, Chen Y, Karin M, and Santoro MG (2000). Anti-inflammatory cyclopentenone prostaglandins are direct inhibitors of IκB kinase. Nature 403:103–108.

Rumi MA, Sato H, Ishihara S, Kawashima K, Hamamoto S, Kazumori H, Okuyama T, Fukuda R, Nagasue N, and Kinoshita Y (2001). Peroxisome proliferator-activated receptor gamma ligand-induced growth inhibition of human hepatocellular carcinoma. Br J Cancer 84:1640–1647.

Sarraf P, Mueller E, Jones D, King FJ, DeAngelo DJ, Partridge JB, Holden SA, Chen LB, Singer S, Fletcher C, and Spiegelman BM (1998). Differentiation and reversal of malignant changes in colon cancer through PPARγ. Nat Med 4:1046–1052.

Schmid RM and Adler G (2000). NF-κB/Rel/IκB: Implication in gastrointestinal diseases. Gastroenterology 118:1208–1228.

Shiraki K, Takase K, and Nakano T (2002). The emerging role of caspase inhibitors in gastrointestinal cancers. J Gastroenterol 37:323–331.

Silke J, Hawkins CJ, Ekert PG, Chew J, Day CL, Pakusch M, Verhagen AM, and Vaux DL (2002). The anti-apoptotic activity of XIAP is retained upon mutation of both the caspase-3 and caspase-9-interacting sites. J Cell Biol 157:115–124.

Sternsdorf T, Puccetti E, Jensen K, Hoelzer D, Will H, Ottmann OG, and Ruthardt M (1999). PIC-1/SUMO-1-modified PML-retinoic acid receptor α mediates arsenic trioxide-induced apoptosis in acute promyelocytic leukemia. Mol Cell Biol 19:5170–5178.

Tamatani M, Che YH, Matsuzaki H, Ogawa S, Okado H, Miyake S, Mizuno T, and Tohyama M (1999). Tumor necrosis factor induces Bcl-2 and Bcl-x expression through NFκB activation in primary hippocampal neurons. J Biol Chem 274:8531–8538.

Tan C and Waldmann TA (2002). Proteasome inhibitor PS-341, a potential therapeutic agent for adult-T cell leukemia. Cancer Res 62:1083–1086.

Thieringer R, Fenyk-Melody JE, Le Grand CB, Shelton BA, Detmers PA, Detmers PA, Somers EP, Carbin L, Moller DE, Wright SD, and Berger J (2000). Activation of peroxisome proliferator-activated receptor γ dose not inhibit IL-6 or TNF-α responses of macrophages to lipopolysaccharide in vitro or in vivo. J Immunol 164:1046–1054.

Thomas WD, Zhnag XD, Franco AV, Nguyen T, and Hersey P (2000). TNF-related apoptosis-inducing ligand-induced apoptosis of melanoma is associated with changes in mitochondrial membrane potential and perinuclear clustering of mitochondria. J Immunol 165:5612–5620.

Toyoda M, Takagi H, Horiguchi N, Kakizaki S, Sato K, Takayama H, and Mori M (2002). A ligand for peroxisome proliferator activated receptor gamma inhibits cell growth and induces apoptosis in human cancer cells. Gut 50:563–567.

Tsubouchi Y, Sano H, Kawahito Y, Mukai S, Yamada R, Kohno M, Inoue K, Hla T, and Kondo M (2000). Inhibition of human lung cancer growth by the peroxisome proliferator-activated receptor-γ agonists through induction of apoptosis. Biochem Biophys Res Commun 270:400–405.

Tsujimoto Y and Shimizu S (2000). Bcl-2 family: Life-or-death switch. FEBS Lett 466:6–10.

Untergasser G, Rumpold H, Plas E, Madersbacher S, and Berger P (2001). A low-molecular-weight fraction of human seminal plasma activates adenylyl cyclase and induces caspase 3-independent apoptosis in prostatic epithelial cells by decreasing mitochondrial potential and Bcl-2/Bax ratio. FASEB J 15:673–683.

Vaidya S, Somers EP, Wright SD, Detmers PA, and Bansal VS (1999). 15-Deoxy-Δ-12,14-prostaglandin J_2 inhibits the β2 integrin-dependent oxidative burst: Involvement of a mechanism distinct from peroxisome proliferator-activated receptor γ ligation. J Immunol 163:6187–6192.

Yabe T, Wilson D, and Schwartz JP (2001). NFκB activation is required for the neuroprotective effects of pigment epithelium-derived factor (PEDF) on cerebellar granule neurons. J Biol Chem 276:43313–43319.

Yang BC, Wang YS, Liu HS, and Lin SJ (2000). Ras signaling in involved in the expression of Fas-L in glioma. Lab Invest 80:529–537.

Zou H, Henzel WJ, Liu X, Lutschg A, and Wang X (1997). Apaf-1, a human protein homologues to *C. elegans* CED-4, participates in cytochrome c-dependent activation of caspase-3. Cell 90:405–413.